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Osteopontin-Induced, Integrin-Dependent Migration of Human Mammary Epithelial Cells Involves Activation of the Hepatocyte Growth Factor Receptor (Met)

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Abstract Osteopontin (OPN) is a secreted glycophosphoprotein which induces migration of mammary carcinoma cells, and has been implicated in the malignancy of breast carcinoma. Hepatocyte growth factor (HGF) induces cell migration of several mammary epithelial cell (MEC) lines, via activation of its cognate receptor (Met). This study examines the mechanism of OPN-induced MEC migration, in terms of the cell surface integrins involved and induction of the HGF/Met pathway. Three different MEC cell lines were used, representing different stages of tumor progression: 21PT, non-tumorigenic; 21NT, tumorigenic; non-metastatic; and MDA-MB-435, tumorigenic, highly metastatic. Human recombinant OPN was found to induce the migration of all three lines. OPN-induced migration of 21PT and 21NT cells was αvβ5 and β1-integrin dependent, and αvβ3-independent, while that of MDA-MB-435 cells was αvβ3-dependent. HGF also induced migration of all three cell lines, and a synergistic response was seen to HGF and OPN together. The increased migration response to OPN was found to be associated with an initial increase in Met kinase activity (within 30 min), followed by an increase in Met mRNA and protein expression. OPN-induced cell migration is thus mediated by different cell surface integrins in MEC lines representing different stages of progression, and involves activation of the HGF receptor, Met. J. Cell. Biochem. 78:465–475, 2000. © 2000 Wiley-Liss, Inc.

Key words: osteopontin (OPN); cell migration; integrin; hepatocyte growth factor (HGF); Met; mammary epithelial cells; breast cancer

Growth, migration, and differentiation of epithelial cells are known to be dependent upon integrin-mediated adhesion to extracellular matrix components [reviews in Assoain, 1997; Bissell, 1999; Gumbiner, 1996]. Similarly, these same cellular processes are also known to be influenced by a number of different growth factor pathways [reviews in Seedorf, 1995; Vande Woude et al., 1997; Heldin, 1998; Birchmeier, 1998]. Several recent studies have addressed the possibility of interactions between integrin and growth factor mediated pathways, with evidence emerging for both growth factor control of cell adhesion events [van der Voort et al., 1997; Weimar et al., 1997; Trusolino et al., 1998; Weimar et al., 1998], and conversely, for integrin-mediated cell adhesion phenomena influencing sensitivity to certain growth factors [Miyamoto et al., 1996; Brooks et al., 1997]. However, the mechanism and biological relevance of these growth factor-integrin interactions are not yet clear.

Our group has particular interest in the role of the secreted glycophosphoprotein OPN in the malignancy of breast cancer. We have found that OPN can induce cell migration and

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invasiveness of cultured mammary epithelial cells (MECs) [Xuan et al., 1994; Xuan et al., 1995; Tuck et al., 1999], that it may be secreted in greater quantity by MECs of greater degree of malignancy [Tuck et al., 1999], and that higher levels (tumor cell or plasma levels respectively) are associated with poorer prognosis in patients with either lymph node negative or metastatic breast cancer [Tuck et al., 1998; Singhal et al., 1997]. Evidence from us [Xuan et al., 1994, 1995; Tuck et al., 1999], and others [Senger et al., 1996], has indicated that OPN-induced cell movement is a directed, RGD-dependent response, although CD44-mediated phenomena may also be involved [Weber et al., 1996; Bourguignon et al., 1998, 1999; Katagiri et al., 1999; Tuck et al., unpublished observations]. Cell adhesion studies have shown for a variety of cell types that the major cell surface integrins involved in OPN binding include $\alpha v\beta 1$, $\alpha v\beta 3$, and $\alpha v\beta 5$ [Hu et al., 1995; Liaw et al., 1995].

Given this information, along with the abundant evidence for the importance of HGF/Met in cell motility of MECs [Bhargava et al., 1992; Rosen et al., 1994; Rahimi et al, 1998], and in the malignancy of breast cancer [Yamashita et al., 1994; Tuck et al., 1996; Yao et al., 1996; Jin et al., 1997; Beviglia et al., 1997; Ghoussoub et al., 1998], we set out to examine the nature of OPN-induced cell migration, with respect to the involvement of cell surface integrins known to bind OPN, and possible interactions with the HGF/Met pathway. We have made use of three MEC lines, of differing malignancy: 21PT, non-tumorigenic; 21NT, tumorigenic, non-metastatic [Band et al., 1990]; and MDA-MB-435: tumorigenic, highly metastatic [Price et al., 1990]. We have assessed these cells for migratory responsiveness to OPN, alone and in combination with HGF. Having found evidence for a synergistic relationship between OPN and HGF in inducing cell migration, we proceeded to characterize the cell surface integrins involved, using blocking antibodies to $\alpha v\beta 5$, $\beta 1$, or $\alpha v\beta 3$ integrins. OPN-treated cells were then examined in time course experiments for induction of Met kinase activity and tyrosine phosphorylation, and for levels of HGF and Met mRNA and protein. Incubation with OPN was found to result in rapid activation of Met (all three cell lines), followed by an increase in Met RNA (all three cell lines) and protein (21PT and 21NT).

This work thus provides evidence that MEC cell lines representative of different stages of progression make use of different cell surface integrins in the migration response to OPN, and that this OPN-induced cell migration may be mediated at least in part by activation of Met.

METHODS

Cell Lines and Culture

The 21T series cell lines (21PT, 21NT) were obtained as a kind gift of Dr. Vimla Band (Dana Farber Cancer Institute) [Band et al., 1990]. These cells were maintained in culture in α-MEM supplemented with 10% FCS, 2 mM L-glutamine (all from GIBCO-BRL/Life Technologies, Grand Island, NY), insulin (1 μg/ml), epidermal growth factor (EGF; 12.5 ng/ml), hydrocortisone (2.8 μM), 10 mM HEPES, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 50 μg/ml gentamycin (all from Sigma; αHE medium). MDA-MB-435 cells [Price et al., 1990] were obtained as a kind gift of Dr. Janet Price (MD Anderson Cancer Center, Houston, TX), and were grown in α-MEM with 10% FCS (both from GIBCO-BRL/Life Technologies).

Cell Migration

Cell migration assays were performed essentially as described previously [Xuan et al., 1995], using 24-well transwell chambers with polycarbonate filters of 8 μm pore size (Costar, Cambridge, MA). Gelatin (Sigma) was applied at 6 μg/filter and air dried. The gelatin was rehydrated with 100 μl of serum-free αHE medium at room temperature for 90 min. Lower wells contained 800 μl of αHE plus 0.1% BSA, with or without OPN, HGF, and/or blocking antibodies (as specified in Figs. 1 and 2). Human OPN (50 μg/ml) used was the full length human recombinant GST-OPN (hrOPN), as previously described [Xuan et al., 1994]. Previous control experiments have shown that the GST portion alone has no influence on migration of these cells. Human HGF (20 ng/ml) was obtained from Collaborative Biomedical Products (Becton-Dickinson, Bedford, MA). Blocking anti-integrin antibodies included anti-$\alpha v\beta 3$ (Cedarlane, Hornby, ON), anti-$\alpha v\beta 5$ (GIBCO-BRL), and anti-$\beta 1$ (GIBCO-BRL), all used at saturating concentrations as determined by preliminary titration experiments. Cells

(5×10^4) were added to each upper well in αHE medium with 0.1% BSA and incubated for 5 h at 37°C. At the end of the incubation time, the cells that had migrated to the undersurface of the filters were fixed in place with gluteraldehyde and stained with hematoxylin. Cells that had not migrated and were attached to the upper surface of the filters were removed from the filters with a cotton swab. The lower surfaces of the filters were examined microscopically under 100× magnification and representative areas were counted to determine the number of cells that had migrated through the filters. Control experiments were also performed in which blocking antibody in the lower chamber was replaced by non-immune mouse IgG (Cedarlane) at comparable concentration.

All cell migration and invasion assays were performed in triplicate. Statistical differences between groups were assessed using Student's *t*-test, with SigmaStat (Jandel Scientific, San Rafael, CA) statistical software.

Immunoprecipitation and Western Blotting for Met and Phosphotyrosine

Cells in monolayer were grown to 85–90% confluence, serum starved overnight, and incubated in serum-free medium either with or without human OPN (50 μg/ml) or HGF (20 ng/ml) for the times specified. Cells were then rinsed with cold PBS, and lysed in lysis buffer containing 50 mM Tris-HCL (pH 7.5), 150 mM NaCl, 1% NP-40, 1 mM Na_3VO_4, 50 mM NaF, 2 mM EGTA, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 mM PMSF. Lysates were centrifuged for 10 min at 14,000 rpm in an IEC/Micromax centrifuge at 4°C. Protein concentration of supernatants was determined using a bicinchoninicacid protein assay (Pierce, Rockford, IL). Equal protein amounts of each lysate were immunoprecipitated with rabbit anti-human Met polyclonal antibody at 4°C for 2 h.

Immunoprecipitates were collected on protein A-Sepharose (Amersham-Pharmacia Bio-

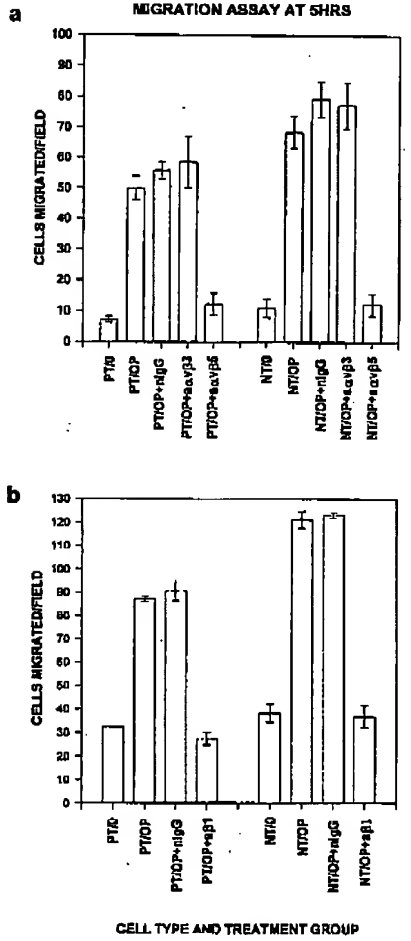

Fig. 1. a: OPN-induced migration of 21PT (PT) and 21NT (NT) cells is αvβ5, not αvβ3, integrin-dependent. Migration assays were performed as described in Materials and Methods. Lower chamber conditions were as follows: 0.1% BSA only (0); 50 μg/ml hrOPN only (OP); 50 μg/ml hrOPN with 15 μg/ml non-specific mouse IgG (OP+nIg); 50 μg/ml hrOPN with 30 μg/ml anti-αvβ3 integrin blocking antibody (OP+αvβ3); or 50 μg/ml hrOPN with 15 μg/ml anti-αvβ5 integrin blocking antibody (OP+αvβ5). b: OPN-induced migration of 21PT (PT) and 21NT (NT) cells is β1 integrin-dependent. Lower chamber conditions were as follows: 0.1% BSA only (0); 50 μg/ml hrOPN only (OP); 50 μg/ml hrOPN with 15 μg/ml non-specific mouse IgG (OP+nIg); or 50 μg/ml hrOPN with 15 μg/ml anti-β1 integrin blocking antibody (OP+aβ1). Bar graphs represent the mean of four or five counts from each of three separate wells; error bars are SEM.

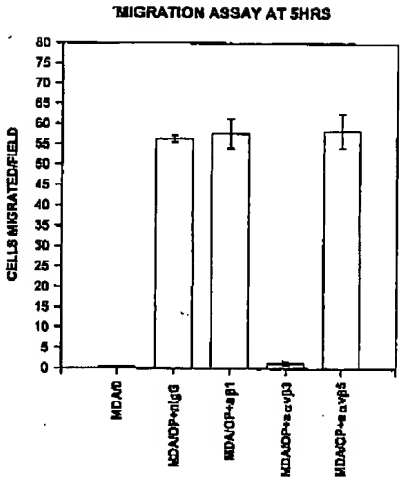

Fig. 2. OPN-induced migration of MDA-MB-435 (MDA) cells is αvβ3, not αvβ5 or β1 integrin-dependent. Migration assays were performed as described in Materials and Methods. Lower chamber conditions were as follows: 0.1% BSA only (0); 50 μg/ml hrOPN only (OP); 50 μg/ml hrOPN with 25 μg/ml non-specific mouse IgG (OP+nIg); 50 μg/ml OPN with 15 μg/ml anti-β1 integrin blocking antibody (OP+aβ1); 50 μg/ml hrOPN with 25 μg/ml anti-αvβ3 integrin blocking antibody (OP+aαvβ3)); or 50 μg/ml hrOPN with 15 μg/ml anti-αvβ5 integrin blocking antibody (OP+aαv5). Bar graphs represent the mean of four or five counts from each of three separate wells; error bars are SEM.

tech, Baie d'Urfe, Quebec, Canada), washed three times with lysis buffer, separated by 7% SDS-PAGE, and transferred to a nitrocellulose membrane. The membrane was blocked for 15 min with 3% skim milk, or 1% BSA, in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20), and probed for 1 h with either mouse anti-human Met (DL-21 clone, Upstate Biotechnology Inc., Lake Placid, NY) or anti-phosphotyrosine antibody (PY20 clone, Transduction Labs, Lexington, KY). The membrane was washed three times for 5 min each with TBST buffer, incubated with horseradish peroxidase-labeled secondary anti-mouse antibody (Amersham-Pharmacia Biotech) for 15 min, and washed three times with TBST for 10 min each. Immune complexes were detected using ECL (Mandel/NEN, Guelph, ON).

In Vitro Met Kinase Assay

Cell cultures incubated in the presence or absence of hrOPN (50 μg/ml) or HGF (20 ng/ml) were rinsed with cold PBS, lysed, and immunoprecipitated as above. Immunoprecipitates were washed twice with cold lysis buffer and once with cold kinase buffer (20 mM PIPES, pH 7.0, 10 mM $MnCl_2$, 10 μM Na_3VO_4). In vitro Met kinase activity was determined by incubating immunoprecipitates with 20 μl of kinase buffer containing 10 μCi [γ-^{32}P] ATP at 30°C for 10 min. The reaction was stopped by addition of 2× SDS sample buffer containing 5% β-mercaptoethanol. Samples were boiled for 3 min and subjected to 7% SDS-PAGE. Serine and threonine phosphorylations were hydrolyzed by incubating the acrylamide gel in 1 M KOH at 45°C for 30 min, followed by fixing in 45% MeOH and 10% acetic acid for 30 min at room temperature and drying for 2 h at 80°C under a vaccum. Autoradiograms were produced and quantitated using a Storm Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Analysis of Met and HGF mRNA Levels

Cell cultures (85–90% confluent) were incubated in serum-free medium for the specified times in the presence or absence of 50 μg/ml hrOPN. Cells were harvested by gentle trypsinization, pelleted, and mechanically homogenized (Polytron PT 1200, Brinkman Instruments [Canada] Ltd., Mississauga, ON). RNA was extracted using TRIzol Reagent (Canadian Life Technologies Inc., Burlington, ON), according to the protocol supplied by the manufacturer. RNA (10 μg/lane) was run on a 1.1% agarose gel with 6.8% formaldehyde, and capillary-transferred to GeneScreen Plus filters (DuPont Canada Inc., Mississauga, ON). Blots were probed with denatured, oligolabeled [α-^{32}P]-dCTP cDNA probes (labeled using a kit provided by Pharmacia), according to the procedures provided by the manufacturers, and as previously described [Tuck et al., 1990, 1991]. cDNA probes were as follows: hepatocyte growth factor (HGF)—540 bp BamHI-XhoI fragment of human HGF cDNA [Nakamura et al., 1989]; Met/HGF receptor (HGFR)—800 bp EcoRI-EcoRV fragment of the human *met* cDNA [Park et al., 1987]; 18S rRNA (18S)—from p100D9.

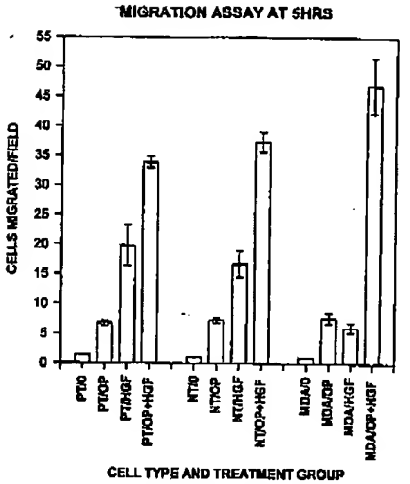

Fig. 3. Synergistic effect of OPN and HGF on migration of 21PT (PT), 21NT (NT), and MDA-MB-435 (MDA) cells. Migration assays were performed as described in Materials and Methods. Contents of the lower chamber consisted of either: medium (αH, no EGF) without HGF or hrOPN (0); medium with 50 μg/ml hrOPN (OP); medium with 10 ng/ml HGF (HGF); or medium with 50 μg/ml hrOPN and 10 ng/ml HGF (OP+HGF). Bar graphs represent the mean of four or five counts from each of three separate wells; error bars are SEM.

RESULTS

OPN-Induced Migration of 21PT and 21NT Cells Involves Different Cell Surface Integrins Than for MDA-MB-435 Cells

Cell migration of 21PT, 21NT, and MDA-MB-435 cells was found to occur in response to hrOPN at a level comparable to that determined previously [Tuck et al., 1999]. Blocking experiments were performed using saturating concentrations of anti-integrin antibodies in the lower chamber of transwells, as described in Methods. For 21PT and 21NT cells, complete blocking of OPN-induced cell migration (to baseline levels) was obtained with the anti-αvβ5 and β1 integrin antibodies (Fig. 1a,b; $P < 0.002$ for all, Student's t-test). In contrast, non-immune mouse IgG did not block migration of either cell line. Saturating concentrations (30 μg/ml) of anti-αvβ3 integrin antibody had no detectable affect on migration of either 21PT or 21NT cells (Fig. 1a).

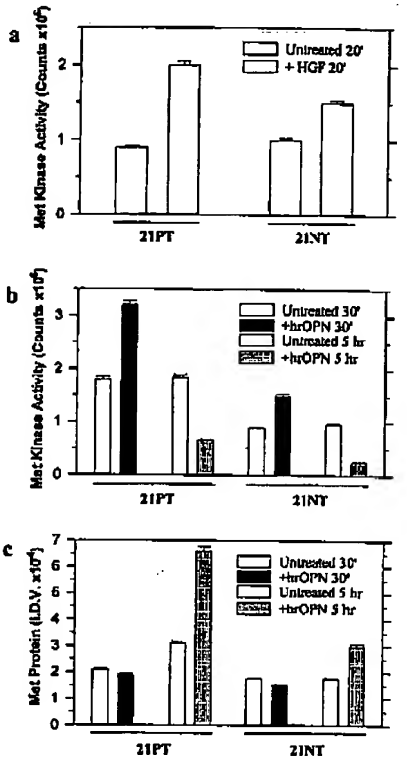

Fig. 4. HGF (a) and OPN (b)-induced increase in total cellular Met kinase activity of 21PT and 21NT cells. OPN (c)-induced increase in total Met protein of 21PT and 21NT cells. a,b: Cells were incubated ±20 ng/ml HGF or 50 μg/ml hrOPN for the times indicated, and cells lysates were prepared. Equal protein amounts of each lysate were immunoprecipitated with anti-Met IgG, and in vitro Met kinase activity was determined as described in Materials and Methods. Quantitation was done using a PhosphorImager. Total Met kinase activity is expressed in cpm/sample. c: Met protein was quantitated by immunoprecipitation with rabbit polyclonal anti-Met antibody, followed by 7% SDS-PAGE and Western blotting as described in Materials and Methods. Total Met protein was quantitated by densitometry and is expressed in integrated density value units. Error bars represent an average standard deviation of 2.5%, as determined by repetitive measurements of individual bands (instrument error). Each graph is representative of at least two separate experiments.

In contrast to results with 21PT and 21NT cells, anti-αvβ5 and β1 integrin antibodies showed no blocking effect on OPN-induced cell migration of MDA-MB-435 cells, when used at the same high concentrations shown to effect complete blocking of 21PT and 21NT responsiveness (15 μg/ml of either anti-integrin antibody; Fig. 2). On the other hand, OPN-induced migration of MDA-MB-435 cells was completely blocked by anti-αvβ3 integrin antibody, at a concentration (25 μg/ml) lower than that which still had no effect on migration of 21PT or 21NT (30 μg/ml; cf. Fig. 1; $P = 0.0008$, Student's t-test).

The OPN-induced migration of the metastatic cell line of this series—MDA-MB-435, thus was found to be αvβ3 integrin-dependent, whereas that of non-metastatic 21NT and 21PT cells was αvβ5 and β1-dependent, αvβ3-independent.

HGF-Induced Cell Migration and Synergistic Effect With OPN

As was found for response to OPN, all three cell lines (21PT, 21NT, MDA-MB-435) showed increased cell migration in response to human recombinant HGF alone (Fig. 3). Combining both HGF and osteopontin in the lower chamber resulted in a degree of cell migration for all three cell lines that was significantly greater than the sum of the isolated HGF and OPN responses (i.e., synergistic; Fig. 3; $P < 0.02$ for all, Student's t-test).

Induction of Met (HGFR) Activity by HGF and OPN

Treatment of 21PT and 21NT cells with either HGF or OPN (Fig. 4a,b) resulted in rapid activation of total Met kinase activity in both instances (after 20 min of HGF stimulation, 30 min of OPN stimulation). For both 21PT and 21NT cells, the increase in total Met kinase activity with OPN treatment (at 30 min) was followed by an increase in Met protein level at 5 h of incubation with OPN (Fig. 4c).

Treatment of MDA-MB-435 cells with HGF or OPN (Fig. 5a) resulted in an increase in specific Met kinase activity after 20 (for HGF) to 30 (for OPN) min of incubation, which was associated with an increased tyrosine phosphorylation of Met as well (Fig. 5b). In contrast with 21PT and 21NT cells, we have not been able to detect an OPN-induced increase in total

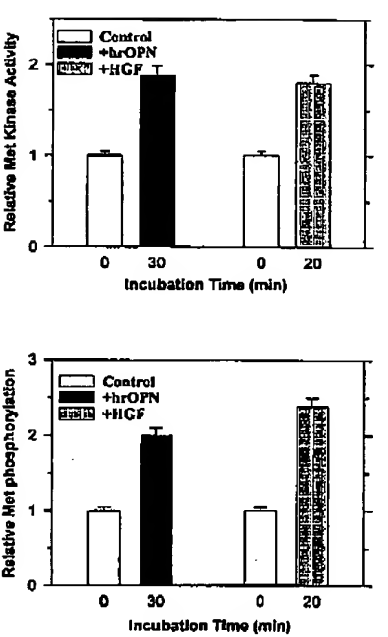

Fig. 5. Induction of specific Met kinase activity (a) and Met tyrosine phosphorlyation (b) of MDA-MB-435 cells by HGF and OPN. MDA-MB-435 cells were incubated ±50 μg/ml hrOPN or 20 ng/ml HGF for the times indicated. Cell lysates were prepared, and equal protein amounts of each lysate were immunoprecipitated with anti-Met IgG. As levels of total Met protein were higher and fluctuated more in MDA-MB-435 cells than in 21PT and 21NT, activation of Met protein in MDA-MB-435 was more appropriately expressed as Relative Met kinase activity (a) and tyrosine phosphorylation (b). a: In vitro Met kinase activity was assayed as described in Materials and Methods. Relative Met kinase activity, normalized to total Met protein, was quantitated using a Phosphorimager. b: Immunoprecipitates were subjected to 7% SDS-PAGE and transferred to nitrocellulose. The membrane was blocked with 1% BSA in TBST, and probed with anti-phosphotyrosine antibody. Detection was performed with HRP-labeled anti-mouse antibody and ECL. Relative Met tyrosine-phosphorylation normalized to total Met protein was quantitated by densitometry. Error bars represent an average standard deviation of 5.0%, as determined by repetitive measurements of individual bands (instrument error). Each graph is representative of at least two separate experiments.

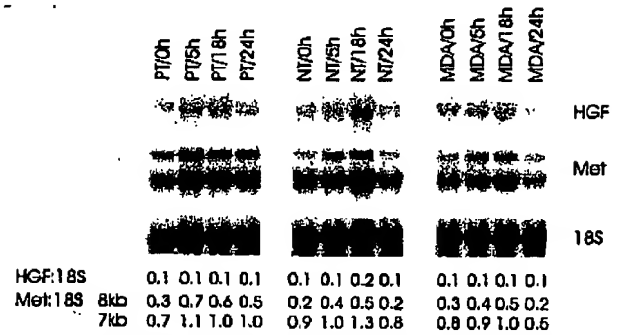

Fig. 6. Time course showing effect of OPN on expression of HGF and Met mRNA by 21PT (PT), 21NT (NT), and MDA-MB-435 (MDA) cells. Near-confluent (85–90%) cell cultures were incubated in serum-free medium with 50 μg/ml OPN for 0, 5, 18, or 24 h. Total RNA (10 μg/lane) was analysed by Northern blotting for expression of HGF (6.0 kb) or Met (HGFR; 8.0 kb [full-length transcript, upper band], 7.0 kb [lower band]). RNA loading and integrity were verified by assessment of 18S rRNA (2.1 kb). Level of HGF and Met (both 8kb and 7kb transcripts) are shown in relation to 18S rRNA, expressed as the ratio of densitometry values for the respective bands (HGF:18S, Met:18S).

Met protein levels in MDA-MB-435 cells, although basal level of Met protein expression in MDA-MB-435 is higher than in 21PT or 21NT (data not shown).

Time Course Showing Effect of OPN on Expression of HGF and Met mRNA

21PT, 21NT, and MDA-MB-435 cells treated with OPN (50 μg/ml) for 0–24 h (Fig. 6), all showed low basal levels of HGF mRNA. Only slight increase in HGF mRNA was detected for 21PT and 21NT, with no appreciable increase for MDA-MB-435 cells (by 18–24 h). In contrast, levels of Met RNA were found to significantly increase in all three cell lines between 5 and 18 h of OPN exposure, falling off by 24 h. Thus, although little change in HGF mRNA was seen after up to 24 h of OPN exposure, significant induction of Met mRNA was seen for all three cell lines.

DISCUSSION

OPN has been implicated in the maligancy of breast cancer in a number of recent studies [e.g., Oates et al., 1996; Singhal et al., 1997; Sung et al., 1998; Tuck et al., 1998, 1999]. It has been shown to be involved in cell adhesion of MECs, and can also induce cell migration in an RGD-dependent fashion [Xuan et al., 1994, 1995; Senger et al., 1996]. The HGF/Met pathway has also been associated with breast cancer malignancy [Yamashita et al., 1994; Tuck et al., 1996; Yao et al., 1996; Jin et al., 1997; Bevigha et al., 1997; Ghoussoub et al., 1998], and is a potent inducer of MEC motility [Bhargava et al., 1992; Rosen et al., 1994; Rahimi et al., 1998]. Here we examine the nature of the integrin response to OPN, in order to establish the specific cell surface integrins involved. We also show that OPN-induced cell migration involves activation of the HGF receptor in a syngergistic fashion with HGF, consistent with cross-talk between integrin and growth factor mediated pathways.

Our discovery that, in a series of breast epithelial cells of differing degrees of malignancy, different cell surface integrins may couple with Met in inducing cell migration is a novel finding. The metastatic member of the series studied, MDA-MB-435 cells, showed the most marked synergy between OPN and HGF in the migration response, and migrated in an αvβ3, not αvβ5 or β1-dependent fashion. In contrast, the non-metastatic cell lines, 21PT and 21NT, migrated in an αvβ5 and β1 dependent, αvβ3-independent fashion. In support of this finding is the work of Wong et al. [1998], who reported that MDA-MB-435 cells express αvβ3 integrin, while less maligant MDA-MB-231 and MCF-7 cells do not (although they all express αvβ5 and

β1). Similarly, van der Pluijm et al. [1997] reported higher expression of αvβ3 in more malignant members of a series of breast carcinoma cell lines. A specific association between αvβ3 expression and breast cancer metastasis has also been reported by Liapis et al. [1996], who detected αvβ3 integrin expression in 100% of breast carcinomas that had metastasized to bone.

This difference in integrin utilization of cells at different stages of progression could affect malignancy in a number of different ways. For example, αvβ3 may be necessary for specific adhesion events vital to invasion and metastasis; a breast cancer cell initially expressing αvβ1 or αvβ5 may require activation of β3 in order to complete that step of the metastatic cascade. Evidence in favor of this scenario (at least in the case of melanoma) comes from the work of Nip et al. [1992], who showed that binding of metastatic cells to lymph node matrix depends on αvβ3 interactions. Alternatively, different integrins may be coupled to different signal transduction pathways, with αvβ3 specifically required for activation of a particular set of genes important in aspects of invasion and metastasis. Ligation of αvβ3 for example, has been shown to induce MMP-2 expression and invasion of melanoma cells [Seftor et al., 1992; Bafetti et al., 1998]. Whether or not different integrins couple to Shc can influence activation of transcription from the Fos serum response element (SRE), affecting responsiveness to growth factors [Wary et al., 1996]. In the case of coupling with the HGF/Met pathway, our work suggests that OPN-induced cell migration via either non-αvβ3 integrins (21PT, 21NT) or αvβ3 integrin (MDA-MB-435) is associated with Met activation, but that the synergistic effect on cell migration is much more pronounced in the cells (MDA-MB-435) expressing αvβ3.

We also found that OPN-induced migration of all three cell lines involves activation of the HGF receptor, Met, with an initial increase in Met activity followed by an increase in Met RNA expression. The kinetics of this effect differ slightly for MDA-MB-435 vs. the 21T series cells. For 21PT and 21NT, a detectable increase in Met protein was also found. Although a similar increase in Met protein levels of MDA-MB-435 cells did not occur, the basal level of Met protein in these cells is quite high, and as specific activity of Met is substantially increased with OPN induction, it is possible that Met turnover is such that protein levels do not further accumulate as they do for the 21T series cells. Regardless, Met is activated by OPN in all three cell lines, and this is associated with increased cell migration. In contrast, HGF mRNA levels were low in all three cell lines, and showed little or no change with OPN treatment. Furthermore, we have not detected increased HGF activity in conditioned media of OPN-treated 21T series or MDA-MB-435 cells (data not shown). Activation of Met by OPN is thus likely due to either an increased sensitivity to trace amounts of ligand present, or to ligand-independent activation. Ligand-independent activation of Met by cellular adhesion has been previously reported for melanoma cells, although the cell surface adhesion receptors involved were not examined [Wang et al., 1996]. Furthermore, integrin binding has been shown to be essential for growth factor (EGF, PDGF, bFGF, IGF-1) induced signal transduction and cell migration [Miyamoto et al., 1996; Brooks et al., 1997]. Reciprocally, HGF can activate cell surface integrins and hence cellular adhesion (and motility) [van der Voort et al., 1997; Weimar et al., 1997, 1998; Trusolino et al., 1998]. Thus, a two-way interaction between integrin and growth factor- mediated pathways likely occurs in the induction of cellular responses such as cell migration.

Multiple points of interaction between signal transduction pathways activated by integrins vs. growth factors have been identified [reviewed in Sastry and Horowitz, 1996; Giancotti, 1997; Swartz, 1997]. Cell attachment can enhance autophosphorylation of growth factor receptors (EGFR, PDGFR, and now Met) in response to their cognate ligands. Integrin binding also has been found to activate phospholipase C (and hence protein kinase C), Raf, and/or MEK in the MAP kinase pathway, and PI-3 kinase in the PI-3K/Rac pathway. All of these pathways are also influenced by growth factors, although the synergistic relationship reported here would suggest that growth factor receptors and integrins may act at different points in the pathway. For example, it has been shown that fibronectin binding to cell surface integrin activates synthesis and supply of phosphatidylinositol 4,5 biphosphate, whereas PDGF receptor controls the activity of phospho-

lipase C [McNamee et al., 1992]. The physical association of integrins and growth factor receptors at the focal adhesion complex (FAC) [Plopper et al., 1995] provides a mechanism by which such cross-talk would be facilitated. Finally, interactions between growth factor and integrin pathways could occur at the level of differential effects on members of the FAC itself [Schlaepfer and Hunter, 1998].

In the case of signal transduction initiated by OPN, ligation of αvβ3 by OPN activates PI-3 kinase in osteoclasts [Hruska et al., 1995]. In an osteoblastic cell line (UMR 106-6), OPN triggers the autophosphorylation of focal adhesion kinase (FAK) [Liu et al., 1997]. In *ras*-transformed fibroblasts, OPN can induce tyrosine phosphorylation of a number of different FAC associated proteins [Lopez et al., 1995]. HGF activation of its receptor, Met, can also stimulate phosphorylation of FAK in some cells, perhaps via $pp60^{src}$ [Chen et al., 1998]. We have shown in this report that OPN is also capable of activating Met. Thus, although our understanding of the signal transduction pathways induced by OPN is yet in early stages, multiple points of potential interaction between the integrin and growth factor receptor pathways involved are already beginning to emerge.

The interactions between different integrin pathways induced by OPN and the HGF/Met growth factor pathway not only helps conceptually in understanding the clinical associations we have observed between OPN, HGF and malignancy, but also provides clues to regulatory processes vital to tumor aggressiveness—prime targets for treatment strategies based on blocking these processes.

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